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Dynamic attachment of *Chlorovirus* PBCV-1 to *Chlorella variabilis*

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ABSTRACT

Chloroviruses infect their hosts by specifically binding to and degrading the cell wall of their algal hosts at the site of attachment, using an intrinsic digesting enzyme(s). *Chlorovirus* PBCV-1 stored as a lysate survived longer than virus alone, suggesting virus attachment to cellular debris may be reversible. Ghost cells (algal cells extracted with methanol) were used as a model to study reversibility of PBCV-1 attachment because ghost cells are as susceptible to attachment and wall digestion as are live cells. Reversibility of attachment to ghost cells was examined by releasing attached virions with a cell wall degrading enzyme extract. The majority of the released virions retained infectivity even after re-incubating the released virions with ghost cells two times. Thus the chloroviruses appear to have a dynamic attachment strategy that may be beneficial in indigenous environments where cell wall debris can act as a refuge until appropriate host cells are available.

Introduction

Chloroviruses (family *Phycodnaviridae*) are large, plaque-forming, dsDNA (290 to 370 kb) containing viruses that infect certain eukaryotic chlorella-like green algae (Dunigan et al., 2006; Yamada et al., 2006; Van Etten and Dunigan, 2012). The chloroviruses have up to 400 protein encoding genes (CDSs), with homologs from both prokaryotes and eukaryotes (Dunigan et al., 2012). The prototype chlorovirus, *Paramecium bursaria* chlorella virus (PBCV-1), infects *Chlorella variabilis* strain NC64A (Blanc et al., 2010; Hoshina et al., 2010). Cryo-electron micrographs and 3D image reconstruction of PBCV-1 virions established that the mature virion is an icosahedral $T=169d$ particle about 1900 Å in diameter (between 5-fold vertices) with an internal membrane (Yan et al., 2005). Recent 5-fold symmetry averaging 3D-reconstruction experiments indicated that one of the PBCV-1 vertices is unique and contains a spike structure that is 560 Å long; with 340 Å protruding from the surface of the virus (Cherrier et al., 2009; Zhang et al., 2011). The part of the spike that is outside the capsid has an external diameter of 35 Å at the tip, expanding to 70 Å at the base. The spike structure widens to 160 Å inside the capsid and forms a closed cavity inside a large pocket between the capsid and the internal membrane enclosing the virus DNA. Each of the PBCV-1 20 trisymmetrons has one fiber attached to a special capsomer.

PBCV-1 initiates infection by specific attachment to the *C. variabilis* cell wall with the spike at the unique vertex oriented toward the cell wall (Meints et al., 1984; Zhang et al., 2011). The fibers probably assist in securing virus attachment to the cell wall (Van Etten et al., 1991). Attachment is immediately followed by host cell wall degradation at the point of contact by a virion-associated enzyme(s). Following host cell wall degradation, the PBCV-1 internal membrane presumably fuses with the host membrane. Rapid depolarization of the host membrane follows (Frohns et al., 2006), probably triggered by a virus-encoded K^+ channel that is located in the virus internal membrane, followed by the rapid release of K^+ from the cell (Neupärtl et al., 2008; Romani et al., 2013). The rapid loss of K^+ and associated water fluxes from the host reduce cellular turgor pressure, which may

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aid ejection of viral DNA and virion-associated proteins into the cell (Thiel et al., 2010). Interestingly the spike is too narrow for DNA to pass into the cell and it likely serves to aid in puncturing the wall before being jettisoned. An empty virus capsid is left attached to the outside of the cell wall. Host membrane depolarization may also prevent infection by a second virus (Greiner et al., 2009).

Although some of the early events in PBCV-1 infection are now understood, not much is known about the initial attachment and the nature of the host receptor. The PBCV-1 receptor in the *C. variabilis* cell wall is distributed evenly over the entire surface and there are at least 5000 binding sites per cell (Meints et al., 1988); additionally, the virus binds efficiently to cell walls alone and can digest the wall at the site of attachment (Meints et al., 1984). The virus receptor is probably a carbohydrate, although this conclusion was based on negative data, i.e., the receptor is unlikely to be a protein(s) because incubation of isolated walls with many proteases had no effect on PBCV-1 attachment. In a previous study we examined the reversibility of PBCV-1 attachment to *C. variabilis* cell walls in the presence of Bold's basal medium (BBM) and tried several non-enzymatic procedures to release infectious virus. However, none of these procedures were successful. Thus it was concluded that attachment was non-reversible (Meints et al., 1988). In the current manuscript we have re-investigated PBCV-1 attachment to its host because, as reported here, PBCV-1 attached to host cell walls retained infectivity longer than free virus.

Results and discussion

Ghost cells

We have often observed that viruses stored as lysates appear to have a longer "shelf-life" compared to highly purified virus preparations. This suggests that there is something in the lysate, possibly cell wall debris that stabilizes the virus. The current study used ghost cells to model virus attachment instead of live cells because they provide certain experimental advantages, e.g., long-term attachment experiments can be conducted and, as described below, the wall can be digested after virus attachment with an enzyme preparation that releases infectious virions. The ghost cells are *C. variabilis* cells extracted with 100% methanol until the chlorophyll is removed. PBCV-1 attaches to ghost cells and isolated *C. variabilis* cell walls at the same rate as actively growing cells and the attached virus digests a hole in the wall fragments (Meints et al., 1988). However, virus DNA is not released under these conditions, presumably because the virus requires a signal from the host to eject DNA. One of the possible triggers of DNA release might be a depolarization of the host plasma membrane, which presumably occurs during fusion of the virus internal membrane with the host membrane.

PBCV-1 survival during long-term storage

To evaluate PBCV-1 stability bound to ghost cells, a time course experiment was conducted. PBCV-1 virus was mixed with *Chlorella* ghost cells and incubated for 96 h. Samples were collected at specified times and virus concentration was assessed by plaque assay. PBCV-1 virus kept under similar conditions but without ghost cells served as a control. However, infectious PBCV-1 was not released from ghost cells after 96 h incubation at room temperature (Fig. 1A). Then the experiment was extended for two months (Fig. 1B) on the assumption that prolonged incubation might result in release of virus attached to the cell walls; the concentration of infectious virus began to decline after 3 weeks. At the conclusion of this study (65 days) infectious virus counts had

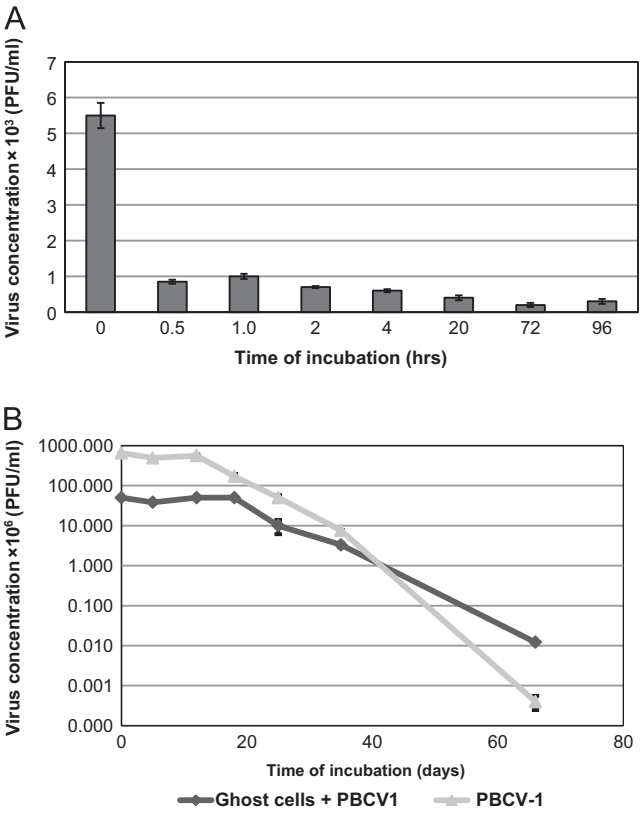

Fig. 1. Attachment of PBCV-1 to ghost cells. A. Chlorella ghost cells in TB at a concentration 5×10^8 cell/ml were mixed with PBCV-1 (10^7 PFU/ml) (MOI 0.02) and incubated for 96 h. The control had an equal amount of PBCV-1 minus ghost cells. B. Long-term virus survival in the presence of host ghost cells. Chlorella ghost cells were mixed with PBCV-1 and kept in the dark at room temperature for 2 months. The control was intact virus kept under the same conditions. Samples were plaque assayed at the designated times. Results are plotted as mean ± standard deviation ($n=4$).

decreased about 6 logs for PBCV-1 alone (control). However, the concentration of infectious PBCV-1 incubated with the ghost cells was 30 times greater than the control.

Virus interactions with surfaces of biological and chemical particles suspended in water have previously been reported to enhance virus survival by protecting them from environmental factors (Gerba, 1984; Templeton et al., 2008). However, this type of protection is not specific. Therefore, these experiments were repeated with two modifications. PBCV-1 was added to a solution of 50 mM Tris–HCl, pH 7.8 (TB) with either bovine serum albumin (BSA) (1 mg/ml) or non-host chlorella ghost cells in order to determine if protection of PBCV-1 was specific. The PBCV-1 survival rate did not increase in the presence of BSA or non-host cells, suggesting that attachment of PBCV-1 to its specific host stabilizes virus survival. This finding suggests that PBCV-1 attachment to cell wall debris is beneficial for long term virus survival, which in nature could provide a selective advantage. These findings prompted us to re-examine PBCV-1 attachment.

Effect of calcium on virus attachment

PBCV-1 binds efficiently to *C. variabilis* (live or ghosts cells) in the culture medium MBBM, but inefficiently to ghost cells in water or several other solutions tested (Meints et al., 1988). Preliminary experiments indicated that a divalent cation, especially calcium, might aid viral entry. To evaluate this possibility, PBCV-1 was added to ghost cells in either TB alone or TB amended with 2 mM $CaCl_2$ (Ca^{++}). Virus attachment was quantified by incubating virus

and ghost cells for 60 min at room temperature. The residual infectious virus that had not bound to ghost cells was then plaque assayed. PBCV-1 was found to attach equally well to ghost cells in TB alone as it did to TB plus 2 mM Ca^{++} (Fig. 2). However, this experiment did not provide any information on whether the attached virions digested a hole in the cell wall. Therefore, transmission electron microscopy (TEM) was used to monitor virus attachment and cell wall degradation. PBCV-1 in TB or TB amended with 2 mM Ca^{++} was added to ghost cells and incubated for 1 h. The samples were fixed, thin sectioned, stained, and examined by TEM. For each treatment, 800 virus particles (8 reps with 100 virions each) were counted in randomly selected fields. The observed virus particles were separated into 3 categories (Fig. 3): category I) viruses attached to the cell wall, but no apparent cell wall degradation; category II) virus particles attached and digested a hole in the cell wall; category III) virus particles not attached to the cell wall ("free" virus particles). PBCV-1 attached to the host walls in both solutions (Fig. 3). However, the number of virus particles in category II was 2-fold more in the presence of Ca^{++} compared to TB alone. These results indicate that PBCV-1 can attach to the host cell wall and digest it in TB; however, Ca^{++} enhances cell wall degradation.

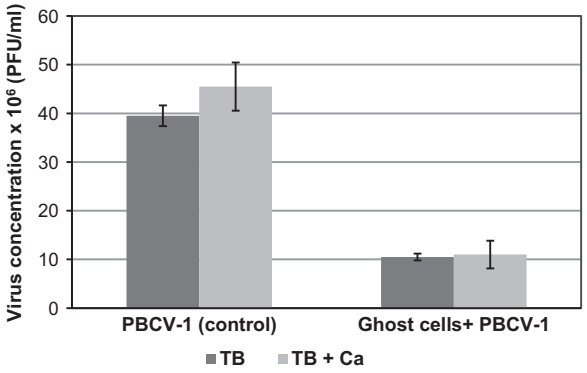

Fig. 2. Effect of calcium on virus attachment. PBCV-1 at 10^8 PFU/ml was added to the ghost cells ($\sim 10^8$/ml) at a MOI of 0.5 either in TB or TB+2 mM $CaCl_2$. Virus concentration was assayed by plaquing after 1 h of incubation at room temperature. The control had an equal amount of PBCV-1 virus kept under similar conditions without ghost cells. Results are plotted as mean ± standard deviation ($n=4$).

Virus release using cell wall degrading enzymes

Although several commercial enzymes were evaluated to determine if they could digest *C. variabilis* cell walls (Meints et al., 1988), none of the available enzymes generated *C. variabilis* protoplasts. However, lysin, an enzyme preparation produced from virus-infected cell lysates, digested the *C. variabilis* cell wall leading to protoplast formation (Fig. 4A). In the experiments involving PBCV-1/*C. variabilis*, we used lysin prepared from *Chlorovirus* ATCV-1 and ATCV-1 infected *Chlorella heliozoae* cells, because lysin preparations often contain residual infectious viruses, which can interfere with the plaquing experiments. ATCV-1 neither attaches to nor infects *C. variabilis* cells; therefore, residual infectious ATCV-1 virions in ATCV-1 lysin do not plaque on *C. variabilis*.

PBCV-1 was incubated with ghost cells in TB with or without Ca^{++} for 60 min at room temperature. Half of the samples were treated with lysin overnight at room temperature and assayed the next day. Control samples were not incubated with lysin. The results of the experiment are shown in Table 1. Essentially 100% infectious PBCV-1 was recovered after lysin treatment of the ghost cells in TB. Approximately 50% of infectious PBCV-1 was recovered after lysin treatment of ghost cells in the presence of Ca^{++}. This result suggests that the presence of Ca^{++} during PBCV-1 attachment to host cell walls and digestion of the cell wall results in the

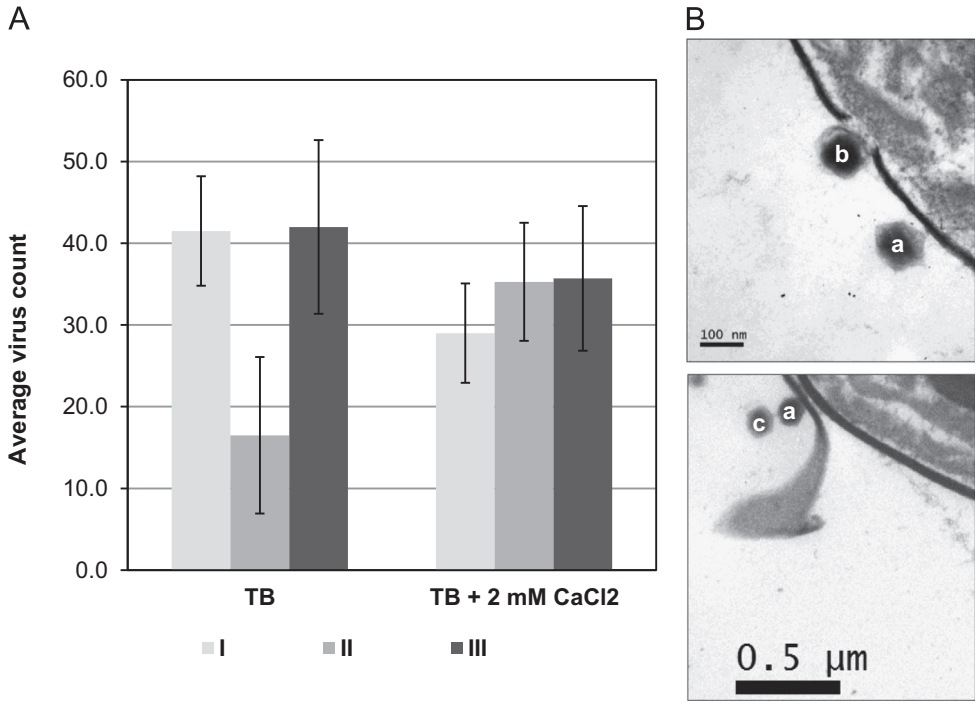

Fig. 3. Effect of calcium on PBCV-1 cell wall digestion (panel A). Ghost cells in TB or TB plus 2 mM $CaCl_2$ were mixed with PBCV-1. After 1 h of incubation the samples were fixed, thin sectioned and examined by TEM. In random selected microscopic fields, 800 virus particles (8 reps with 100 virions each) were counted for each treatment. Virus particles were separated into 3 categories (panel B): a) virus particles attached to the cell wall, but not creating a hole (category I, light gray); b) virus particles digesting a hole in the cell wall (category II, dark gray); c) virus particles not attached to the cell wall ["free" viruses] (category III, black). Results are plotted as mean ± standard deviation ($n=8$).

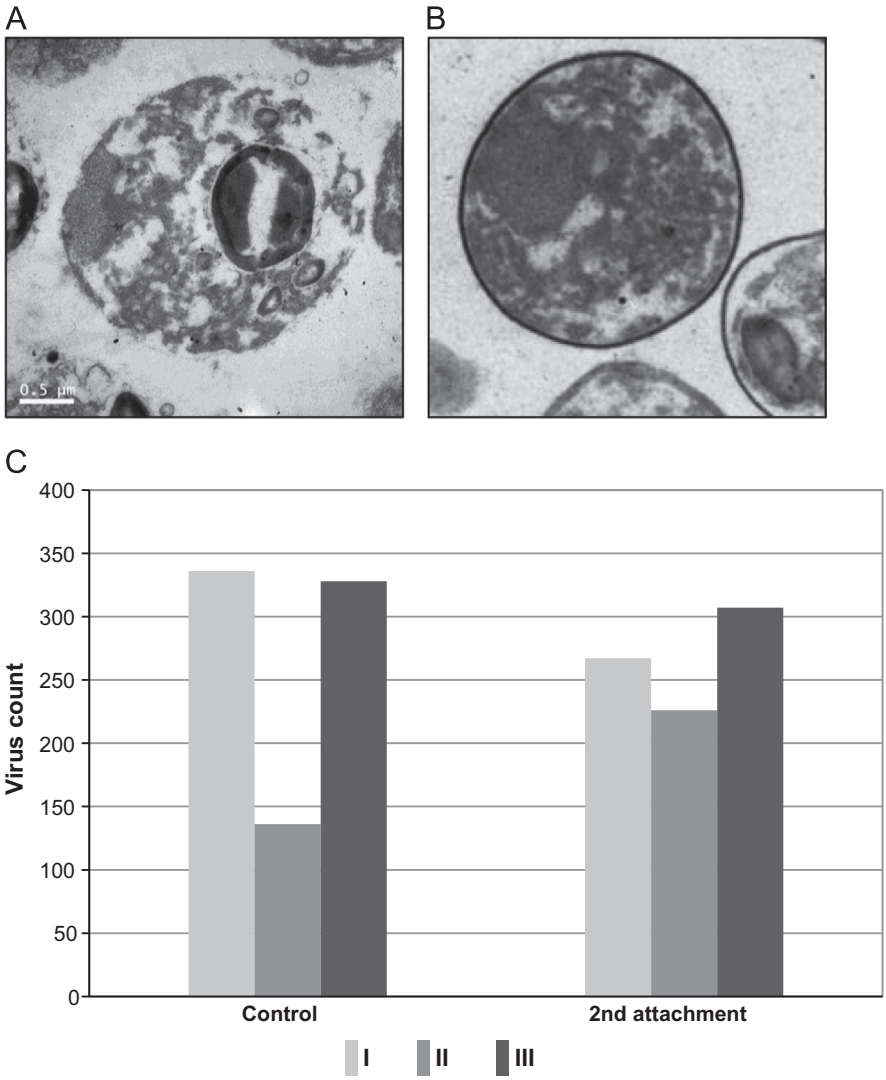

Fig. 4. Ghost *C. variabilis* protoplast generated by overnight lysin treatment (panel **A**) and untreated ghost cells (panel B). **C.** TEM results of PBCV-1 re-attached to the *C. variabilis* ghost cells. Ghost cells were mixed with PBCV-1 and incubated for 1 h then a part of ghost cells was fixed and virus distribution was recorded. The other part of ghost cells was centrifuged briefly to remove unattached virus (category III) then viruses attached to ghost cells (categories II and III) were released by incubating overnight with lysin; the released virus was concentrated by centrifugation, suspended and again mixed with ghost cells. In random selected microscopic view fields, 800 virus particles were recorded for each attachment. Virus particles were separated into 3 categories: a) virus particles attached to the cell wall, but not creating a hole (category I, light gray); b) virus particles digesting a hole in the cell wall (category II, dark gray); c) virus particles not attached to the cell wall (category III, black). Results are plotted as mean $\pm$ standard deviation ($n=8$).

progression of infection into a more advanced phase for some viruses, i.e., a point of no return.

PBCV-1 attachment, release and re-attachment

Could PBCV-1 remain infectious if it is attached, released, re-attached and re-released? To investigate this question PBCV-1 was added to ghost cells and incubated for 1 h. Part of the sample served as a control and the other part of ghost cells was centrifuged briefly to remove unattached ("free") virus then was treated with lysin to release virus from the cell walls; the released virus was added to ghost cells a second time and attachment and cell wall degradation was monitored by TEM. In randomly selected microscopic fields, 800 virus particles (8 reps with 100 virions each) were counted at each step (Fig. 4C). Virus particles were separated into the same 3 categories described above. After the first attachment the distribution of viruses among categories I, II, and III was 42%, 17% and 41%, respectively. After the second attachment the major changes were recorded in categories I and II.

Table 1
Dynamic attachment of PBCV-1 to *C. variabilis* ghost cells after lysin treatment.

	Mean virus concentration ($\times 10^6$ PFU/ml)			
	PBCV-1 (control)	PBCV-1+lysin (control 2)	ghost cells+ PBCV-1	ghost cells+ PBCV-1+lysin
TB	39.5	54.0	10.5	50.0
STDEV ($n=4$)	2.1	2.8	0.7	2.8
TB+Ca++	45.5	50.0	11.0	25.5
STDEV ($n=4$)	4.9	2.8	2.8	0.7

The percent of virus particles decreased in category I (to 33.4% from 42%) and increased in category II (to 28.3% from 17%). The percentage of virus in category III slightly decreased (to 38.4%

from 41%) even though that population of virions was removed from the pool of viruses after the first attachment by brief centrifugation.

Based on the observed data a model with the assumption that viruses can attach and create a hole only once was created (Fig. 5A, Table S1); i.e., only viruses from category I (after the first attachment) can contribute to category II in the second attachment. The modeled distribution of virus particles into the 3 categories was compared with observed results using the chi-squared test (Chernoff and Lehmann, 1952). As reported in Table S1 the chi-squared results equaled 32.854 and the *P* value equaled 0.0001. A small *P*-value (conventionally accepted <0.05) indicates that the observed data were not sampled from the modeled distribution. Thus the virus distribution into the modeled categories did not fit the observed results and this model was rejected.

The observed results best fit a model when virus from both categories (I and II) contributes into each group in the second attachment (Fig. 5B, Fig. 6, Table S1). The difference between observed and predicted values was not statistically significant: the chi-squared value equaled 0.002 and the two-tailed *P*-value equaled 0.9992. The results of the chi-squared test indicated that the observed distribution of virus in the second round of attachment results from about 28% of virions retaining attachment and cell wall digestion activity after previously digesting the wall (Fig. 5B).

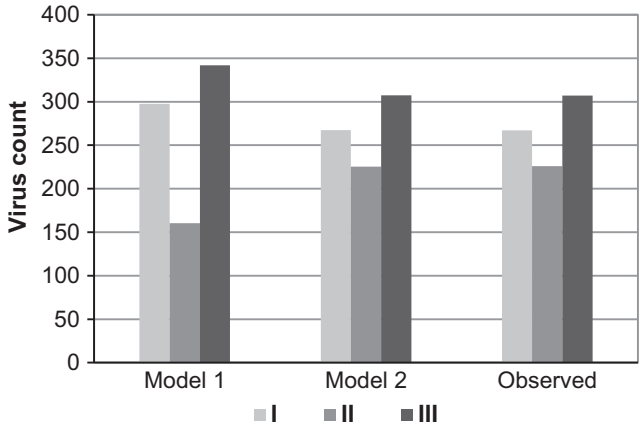

Fig. 6. Hypothetical distribution of viruses into 3 attachment categories. Model 1: distribution based on the assumption that a virus can only digest a hole one time. Model 2: distribution based on the assumption that categories I and II contributes into each group in the second attachment experiment. Category I (light gray): virus particles attached to the cell wall, but not creating a hole; category II (dark gray): virus particles digesting a hole in the cell wall; category III (black): virus particles not attached to the cell wall.

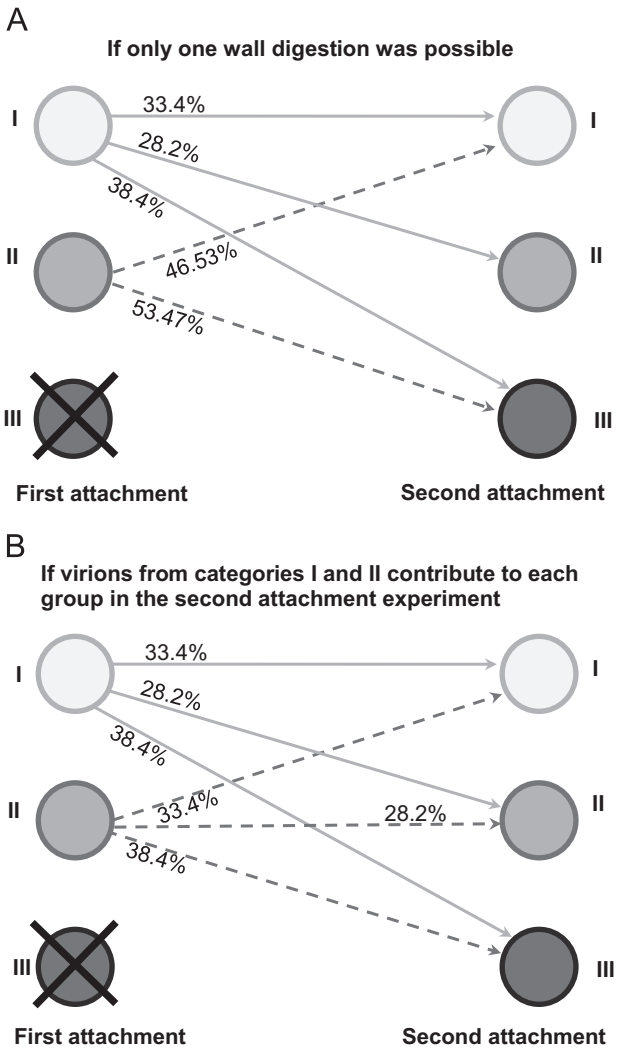

Fig. 5. Hypothetical distribution of viruses into 3 attachment categories. A) Model 1: distribution based on the assumption that a virus can only digest a hole one time (Table 2, Fig. 6). B) Model 2: distribution based on the assumption that virus from both categories (I and II) contributes into each group in the second attachment experiment (Table 2, Fig. 6). Category III particles were removed by centrifugation after the first attachment.

Virus structure after cell wall attachment

A portion of the trisymmetrons around the unique vertex appears to disassemble during virus attachment and cell wall digestion, as observed with at 8.5 Å resolution cryo-electron microscopy reconstruction of PBCV-1 in the presence of *C. variabilis* cell walls (Zhang et al., 2011). This finding would seem to conflict with our observation that PBCV-1 can attach and digest the host cell wall more than once. To investigate possible changes in PBCV-1 structure after attachment and release, viruses were attached to ghost cells in the presence of 2 mM Ca^{++} and after 1 h incubation the sample was centrifuged at low speed to remove unattached virions. To remove any additional unattached virions, the re-suspended pellet fraction (ghost cells with attached virus) was placed on top of a 20% sucrose cushion and centrifuged. After centrifugation, the ghost cell pellet fraction was re-suspended in TB and incubated overnight with lysin. After ghost cell wall digestion, the sample was centrifuged at low speed and the supernatant fraction containing released virus was collected. This sample, which contained virus particles that had been attached and released from ghost cells, was centrifuged on a 10–40% sucrose density gradient. The virions separated into 5 bands (Fig. 7A) containing different amounts of virus. Each band was collected, concentrated by high-speed centrifugation and re-suspended in equal amounts of TB. DNA and protein content of each virus fraction were measured and virus infectivity was determined by plaque assay. These values were compared to a PBCV-1 control sample (Table 2).

The top band (T1), which represented 6% of the total virus particle population, had a low DNA/protein ratio and specific infectivity (i.e., plaque-forming particles/total particles) was ~35 times lower than the PBCV-1 control. Cryo-electron microscopy of the fraction indicated that the particles consisted primarily of DNA free capsids that still contained a membrane (Fig. 7B).

Fractions T2 and T3 contained the two major bands comprising 67% of the total virus particle population. Cryo-electron microscopy images of these fractions (Fig. 7C and D) revealed particles that resembled control virions. However, the T3 population had more empty capsids than the T2 fraction. The specific infectivity of

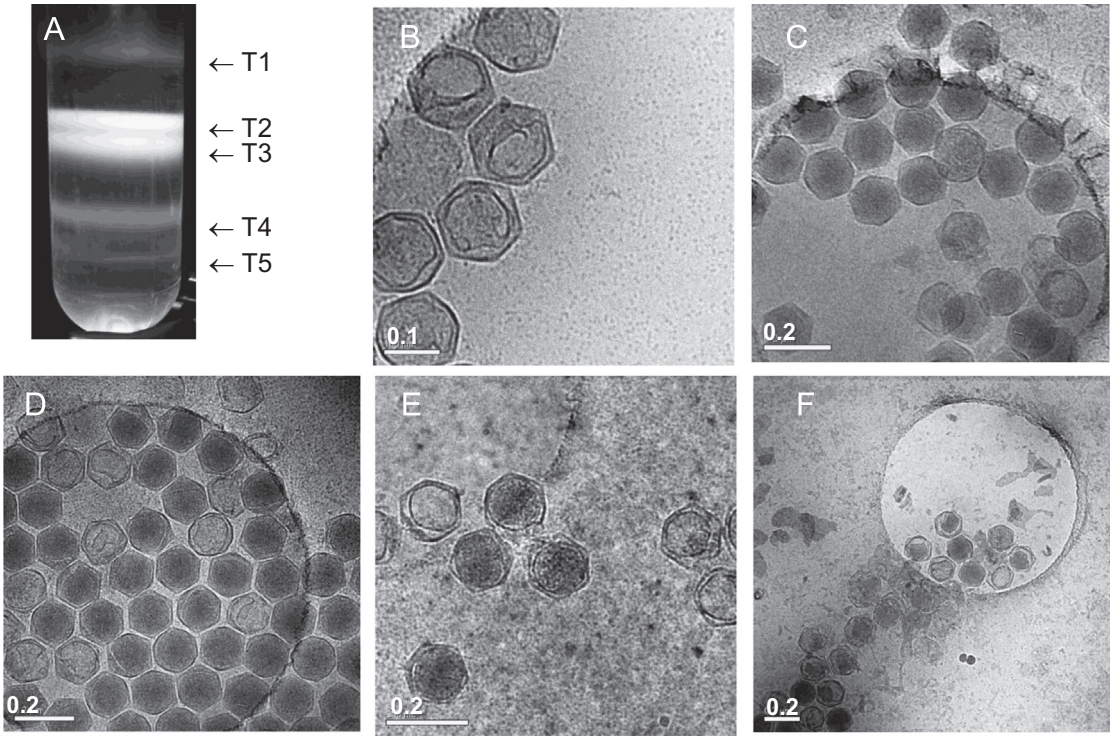

Fig. 7. The cryo-electron microscopic images of different fractions of virus particles after attachment and release from ghost cell walls. A) Separation of previously attached and released PBCV-1 on a sucrose (10–40%) density gradient into 5 layers. B), C), D), E), F) Virus particles from layers T1 to T5, respectively, are from virus fractions depicted in panel A.

Table 2
Properties of virus fractions after release from ghost cell walls and separation by sucrose density gradient centrifugation.

Density gradient band	Nucleic acid (DNA μg/ml)	Protein A280, (mg/ml)	DNA/protein ratio	Virus infectivity (PFU/ml)		Yield distribution (%)[b]	Relative specific infectivity distribution[c]
				Expected[a] ($\times 10^{11}$)	Observed ($\times 10^{11}$)		
T1	171.8	3.37	0. 051	0.35	0.01	5.6	1
T2	852	11.7	0.072	1.55	3.8	27.5	74.3
T3	1227	18	0.068	2.5	2.0	39.6	27.2
T4	694	9.6	0.072	1.25	0.12	22.4	2.8
T5	152.9	2.0	0.076	0.31	0.02	4.9	2.2
Control	501	7.31	0.069	1.0	1.0	N/A	

[a] Virus concentration was calculated according to Van Etten et al. (1983a).
[b] Calculations of yield distribution were made based on summed DNA content for all sucrose density gradient fractions (T1–T5), see Fig. 7.
[c] Based on DNA: Observed[virus]/[DNA]=Specific infectivity. Normalized to the minimum mean value (T1 fraction).

the virions in both fractions was higher than the control (Table 2). We have often noticed that the specific infectivity of PBCV-1 treated with lysin is higher than purified PBCV-1 (e.g., the results in Table 1). This result suggests that lysin digests residual host receptor material from purified virions that block virus infection.

Fraction T4 comprised 22% of the virus particle population with no significant changes in the DNA/protein ratio compare to the control PBCV-1, but the virus specific infectivity was ~10 times lower than for the control virions. There was a noticeable conformation change in the internal membrane of the particles from a nearly icosahedral to a spherical shape (Fig. 7E). Some empty viral particles without DNA were also present.

The lowest band, T5, had a higher DNA to protein ratio compared to the control. The specific infectivity of this fraction was ~15 times lower than the control virions. Cryo-electron microscopy analysis was hampered due to aggregation of the virus particles. The non-aggregated portion of the virus particles is reported in Fig. 7F.

Conclusions

Viruses have many ways to attach to their host cells and initiate infection. The chloroviruses are unique among viruses that infect eukaryotic organisms in that the early stages of infection resemble bacteriophage infection. That is, they attach to their host cell wall, digest the wall at the point of attachment and, after fusion of the host membrane with the internal virus membrane, release DNA and proteins into the cell. The empty virus capsid remains attached to the outside of the wall. The surprising finding in the current study was that PBCV-1 could attach to its host cell wall and then infectious virus could be released from the wall, by an enzyme that was able to digest the cell wall. In fact, infectious PBCV-1 could attach and be released from host cell walls at least twice. This is surprising because one might expect the initial attachment and wall degrading process to inactivate the virus so that it would be non-infectious (Zhang et al., 2011). In fact, this

transition to a point-of-no-return is common for bacteriophages (Molineux, 2006), and occurs with PBCV-1 (Zhang et al., 2011) at a more advanced phase of infection.

A second unexpected finding in the current study was that PBCV-1 infectivity was more stable when the virus was attached to host cell wall fragments than when it was a free particle. Whereas the specific infectivity of standard gradient purified PBCV-1 was estimated to be 0.25 (Table 2, expected), similar to the value previously reported (Van Etten et al., 1983a), the specific infectivity for fraction T2 was more than two times higher; i.e., the specific infectivity of these particles was enriched. This strongly suggests that PBCV-1 is more stable when associated with its host cell wall materials and that viruses released from cell walls through a lysin-like activity are likely to be infectious. This "protection" would be beneficial for long-term virus survival under native conditions. How infectious virus would be released after being attached to wall fragments for a long time is unknown. One possibility is that eventually traces of lysin or unidentified factors facilitate release, allowing the virus to re-enter the water column.

Similar experiments were conducted with two other chloroviruses, CVM-1 and ATCV-1 that infect different green algae. Attachment to their hosts was also reversible (Agarkova, unpublished results). Thus dynamic attachment appears to be characteristic of the chloroviruses.

Materials and methods

Strains and culture conditions

The algal hosts *Chlorella variabilis* (NC64A) and *Chlorella heliozoae* (SAG 3.83), were grown in Bold's basal medium (BBM) modified by the addition of 0.5% sucrose and 0.1% peptone (MBBM) (Van Etten et al., 1983). The production, purification and plaque assay of viruses have been described elsewhere (Agarkova et al., 2006; Van Etten et al., 1983, 1983a).

Preparation of ghost cells

Chlorella cells grown to a concentration of 1–1.5 $\times$ 10^7 cells/ml, were harvested by centrifugation and washed with TB. Cells were extracted with methanol until the chlorophyll was removed and then kept in methanol at 4 °C (ghost cells). To remove traces of methanol before an experiment the ghost cells were washed three times with TB and re-suspended in an appropriate medium.

Virus attachment assay

C. variabilis ghost cells in TB at a concentration of 5–6 $\times$ 10^7 per ml were mixed with PBCV-1 at a designated multiplicity of infection (MOI). Samples were collected at specified times and briefly centrifuged (1000*g* for 1 min) to remove ghost cells with attached virus. Virus concentrations were assessed by plaque assay.

Chlorella ghost cells mixed with PBCV-1 were concentrated by centrifugation after 1 h incubation at room temperature and fixed with a cacodylate-buffered (pH 6.8) 2% glutaraldehyde, 2% formaldehyde (freshly prepared from paraformaldehyde) solution. After washing in buffer, samples were post-fixed in OsO_4 (2% in the same buffer), dehydrated in a graded acetone series, and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were obtained with diamond knives, post-stained with uranyl acetate and lead citrate and examined with a Zeiss 109 TEM. For each treatment, 800 virions (8 reps with 100 virions each) were counted in randomly selected microscopic fields.

Lysin isolation from virus ATCV-1 infected *Chlorella heliozoae* lysates and activity assays were done as described (Agarkova et al., 2008; Kennedy, 1987). The specific activity of lysin (fluorometric units, FU) was defined as the amount of chlorophyll released per 1 μg of protein (Kennedy, 1987). To remove residual virus particles in the lysin extract, the preparation (4 ml) was layered on a 10–40% linear sucrose density gradient, equilibrated with TB and centrifuged in a swinging bucket rotor at 4 °C for 20 min at 72,000*g*. The top portion of the gradient (~10 ml) was collected, dialyzed for 24 h with 3 changes of 25 mM KOH, 50 mM MOPS (pH 7) buffer. The dialysate was concentrated to 5000 FU/ml using Amicon Ultra-Centrifugal Filter Units (3000 NMWL).

Release of viruses attached to the Host cell walls

Viruses attached to chlorella ghost cell walls were released by digesting the ghost walls with lysin (500 FU/ml) overnight, then briefly centrifuged (1 min) at 2300*g* to separate released viruses from cell debris. *C. variabilis* ghost cells were digested with lysin prepared from ATCV-1 infected *C. heliozoae* (Table 1).

Isolation of attached and then released virus for TEM

Viruses were added to ghost cells at a MOI of 100 in the presence of 2 mM Ca^{++} in TB and incubated for 1 h. Ghost cells with attached viruses were centrifuged at low speed (3K for 1 min) to remove unattached viruses, the supernatant fraction was discarded and the pellet fraction was re-suspended in TB. The re-suspended sample was centrifuged through a 20% sucrose pad to remove any unattached virus that co-pelleted with the ghost cells. The pellet fraction containing ghost cells with virus attached to them was re-suspended in TB and treated with lysin in the presence of 2 mM Ca^{++}. The released virus was separated from ghost cells by low speed centrifugation and virus in the supernatant fraction was concentrated by high-speed centrifugation (18,000*g* for 1 h) and re-suspended in TB.

Virus particles that were attached and released from ghost cells were centrifuged on a 10–40% sucrose density gradient made in TB. Visible virus fractions were separated into 5 bands. Each band was collected separately, concentrated by centrifugation (18,000*g* for 1 h) and re-suspended in equal amounts of 250 μl of TB. DNA and protein content of each virus fraction were measured using a NanoDrop 1000 spectrophotometer and virus infectivity was determined by plaque assay.

Model of virus attachment and release

To evaluate differences between observed and expected data the chi-squared statistical test (χ^2) and the *P* values were calculated using free online software from GraphPad (GraphPad Software, Inc., La Jolla, CA). The differences between mean values were considered significant if the *P*-value was less than 0.05.

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Appendix A. Supporting information

Supplementary data associated with this paper can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.002.

References

Agarkova, I., Dunigan, D., Gurnon, J., Greiner, T., Barres, J., Thiel, G., Van Etten, J.L., 2008. Chlorovirus-mediated membrane depolarization of *Chlorella* alters secondary active transport of solutes. J. Virol. 82, 12181–12190.

Agarkova, I.V., Dunigan, D.D., Van Etten, J.L., 2006. Virion-associated restriction endonucleases of chloroviruses. J. Virol. 80, 8114–8123.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J., Salamov, A., Terry, A., Yamada, T., Dunigan, D.D., Grigoriev, I., Claverie, J.-M., Van Etten, J.L., 2010. The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell 22, 2943–2955.

Chernoff, H., Lehmann, E.L., 1952. The use of maximum likelihood estimates in χ^2 tests for goodness-of-fit. Ann. Math. Stat. 25, 576–586.

Cherrier, M.V., Kostyuchenko, V.A., Xiao, C., Bowman, V.D., Battisti, A.J., Yan, X., Chipman, P.R., Baker, T.S., Van Etten, J.L., Rossmann, M.G., 2009. An icosahedral algal virus has a complex unique vertex decorated by a spike. Proc. Natl. Acad. Sci. USA 106, 11085–11089.

Dunigan, D.D., Cerny, R.L., Bauman, A.T., Roach, J.C., Lane, L.C., Agarkova, I.V., Wulser, K., Yanai-Balser, G.M., Gurnon, J.R., Vitek, J.C., Kronschnabel, B.J., Jeannard, A., Blanc, G., Upton, C., Duncan, G.A., McClung, O.W., Ma, F., Van Etten, J.L., 2012. *Paramecium bursaria* chlorella virus 1 proteome reveals novel architectural and regulatory features of a giant virus. J. Virol. 86, 8821–8834.

Dunigan, D.D., Fitzgerald, L.A., Van Etten, J.L., 2006. Phycodnaviruses: a peek at genetic diversity. Virus Res. 117, 119–132.

Frohns, F., Käsmann, A., Kramer, D., Schäfer, B., Mehmel, M., Kang, M., Van Etten, J.L., Gazzarrini, S., Moroni, A., Thiel, G., 2006. Potassium ion channels of chlorella viruses cause rapid depolarization of host cells during infection. J. Virol. 80, 2437–2444.

Gerba, C.P., 1984. Applied and theoretical aspects of virus adsorption to surfaces. Adv. Appl. Microbiol. 30, 133–168.

Greiner, T., Frohns, F., Kang, M., Van Etten, J.L., Käsmann, A., Moroni, A., Hertel, B., Thiel, G., 2009. Chlorella viruses prevent multiple infections by depolarizing the host membranes. J. Gen. Virol. 90, 2033–2039.

Hoshina, R., Iwataki, M., Imamura, N., 2010. *Chlorella variabilis* and *Micractinium reisseri* sp. *nov.* (*Chlorellaceae*, *Trebouxiophyceae*): Redescription of the endosymbiotic green algae of *Paramecium bursaria* (*Peniculia*, *Oligohymenophorea*) in the 120th year. Phycol. Res. 58, 188–201.

Kennedy, J.E., 1987. (Masters Thesis). Purification and Characterization of a Lytic Enzyme From Viral Infection of *Chlorella*-like Green Algae. University of Nebraska, Lincoln.

Meints, R.H., Burbank, D.E., Van Etten, J.L., Lamport, D.T., 1988. Properties of the chlorella receptor for the virus PBCV-1. Virology 164, 15–21.

Meints, R.H., Lee, K., Burbank, D.E., Van Etten, J.L., 1984. Infection of a chlorella-like alga with the virus, PBCV-1: ultrastructural studies. Virology 138, 341–346.

Molineux, I.J., 2006. Fifty-three years since Hershey and Chase; much ado about pressure but which pressure is it? Virology 344, 221–229.

Neupärtl, M., Meyer, C., Woll, I., Frohns, F., Kang, M., Van Etten, J.L., Kramer, D., Hertel, B., Moroni, A., Thiel, G., 2008. Chlorella viruses evoke a rapid release of K^+ from host cells during early phase of infection. Virology 372, 340–348.

Romani, G., Piotrowski, A., Hillmer, S., Gurnon, J., Van Etten, J.L., Moroni, A., Thiel, G., Hertel, B., 2013. A virus-encoded potassium ion channel is a structural protein in the chlorovirus *Paramecium bursaria* chlorella virus 1 virion. J. Gen. Virol. 94, 2549–2556.

Spurr, A.R., 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43.

Templeton, M.R., Andrews, R.C., Hofmann, R., 2008. Particle-associated viruses in water: impacts on disinfection processes. Crit. Rev. Environ. Sci. Technol. 38, 137–164.

Thiel, G., Moroni, A., Dunigan, D., Van Etten, J.L., 2010. Initial events associated with virus PBCV-1 infection of *Chlorella* NC64A. In: Lüttge, U., Beyschlag, W., Büdel, B. (Eds.), Progress in Botany. Springer-Verlag, Berlin, pp. 169–183.

Van Etten, J.L., Burbank, D.E., Kuczmarski, D., Meints, R.H., 1983. Virus infection of culturable chlorella-like algae and development of a plaque assay. Science 219, 994–996.

Van Etten, J.L., Burbank, D.E., Xia, Y., Meints, R.H., 1983a. Growth cycle of a virus, PBCV- 1, that infects chlorella-like algae. Virology 126, 117–125.

Van Etten, J.L., Dunigan, D.D., 2012. Chloroviruses: not your everyday plant virus. Trends Plant Sci. 17, 1–8.

Van Etten, J.L., Lane, L.C., Meints, R.H., 1991. Viruses and virus-like particles of eukaryotic algae. Microbiol. Rev. 55, 586–620.

Yamada, T., Onimatsu, H., Van Etten, J.L., 2006. Chlorella viruses. In: Maramorosch, K., Shatkin, A.J. (Eds.), Adv. Virus Res.. Elsevier Inc., pp. 293–366.

Yan, X., Bowman, V., Olson, N.H., Gurnon, J.R., Van Etten, J.L., Rossmann, M.G., Baker, T.S., 2005. The structure of a $T=169d$ algal virus, PBCV-1, at 15 Å resolution. Microsc. Microanal. 11, 1056–1057.

Zhang, X., Xiang, Y., Dunigan, D.D., Klose, T., Chipman, P.R., Van Etten, J.L., Rossmann, M.G., 2011. Three-dimensional structure and function of the *Paramecium bursaria* chlorella virus capsid. Proc. Natl. Acad. Sci. USA 108, 14837–14842.